Journal of Insect Physiology xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*

Humberto Boncristiani [a,b], Robyn Underwood [c,1], Ryan Schwarz [a], Jay D. Evans [a], Jeffery Pettis [a], Dennis vanEngelsdorp [c,*]

[a] USDA-ARS Bee Research Lab, BARC-East Bldg. 476, Beltsville, MD 20705, USA
[b] Department of Biology, Univ. North Carolina at Greensboro, 312 Eberhart Bldg. Greensboro, NC 27403, USA
[c] Department of Entomology, 4112 Plant Sciences Building, University of Maryland, College Park, MD 20742, USA

ARTICLE INFO

Article history:
Received 30 August 2011
Received in revised form 20 December 2011
Accepted 21 December 2011
Available online xxxx

Keywords:
Acaricides
Honey bee
Gene expression
Varroa
Sublethal effects

ABSTRACT

The effect of using acaricides to control varroa mites has long been a concern to the beekeeping industry due to unintended negative impacts on honey bee health. Irregular ontogenesis, suppression of immune defenses, and impairment of normal behavior have been linked to pesticide use. External stressors, including parasites and the pathogens they vector, can confound studies on the effects of pesticides on the metabolism of honey bees. This is the case of *Varroa destructor*, a mite that negatively affects honey bee health on many levels, from direct parasitism, which diminishes honey bee productivity, to vectoring and/or activating other pathogens, including many viruses. Here we present a gene expression profile comprising genes acting on diverse metabolic levels (detoxification, immunity, and development) in a honey bee population that lacks the influence of varroa mites. We present data for hives treated with five different acaricides; Apiguard (thymol), Apistan (tau-fluvalinate), Checkmite (coumaphos), Miteaway (formic acid) and ApiVar (amitraz). The results indicate that thymol, coumaphos and formic acid are able to alter some metabolic responses. These include detoxification gene expression pathways, components of the immune system responsible for cellular response and the c-Jun amino-terminal kinase (JNK) pathway, and developmental genes. These could potentially interfere with the health of individual honey bees and entire colonies.

1. Introduction

Over the last 5 years, high overwinter mortality of honey bee, *Apis mellifera*, colonies (vanEngelsdorp et al., 2008, 2010a; vanEngelsdorp and Meixner, 2010) and losses of native pollinators have been documented in North America and Europe (Faucon et al., 2002; Biesmeijer et al., 2006; Neumann and Carreck, 2010). Such declines have implications for both the beekeeping industry and for those producers who rely on bees for pollination of their crops. Colony Collapse Disorder (CCD), one possible cause of this decline, has only been confirmed in the US. This disorder is characterized by a rapid loss of the adult bee population leaving no dead bees in the colony or apiary. When colonies are in the last stages of collapse, the queen is present with a small group of apparently newly emerged adult bees and large amounts of unattended brood. Collapsing colonies do not have excessive populations of *Nosema* sp. or of the parasitic mite, *Varroa destructor*. There is agreement among beekeepers and scientists that no one factor alone is responsible for the dramatic losses of honey bees in general or CCD specifically (vanEngelsdorp et al., 2009).

Most honey bee losses from 1966 to 1979 were attributable to pesticides (Atkins, 1975). Nowadays, many other causes for colony losses have been proposed including pathogens (Chen and Siede, 2007; Cornman et al., 2009; Fries, 2010; Di Prisco et al., 2011) and parasitic mites (Boecking and Genersch, 2008). Varroa mites are considered to be the most destructive pest of honey bees worldwide. The role of pesticides in honey bee colony losses, with their sub-lethal and synergistic effects, has recently regained consideration (Mullin et al., 2010), and is the subject of an increasing number of studies (Haynes, 1988; Thompson, 2003).

Even low levels of pesticides can be problematic. For example, sub-lethal levels of neonicitinoids have been shown to impair the learning abilities of honey bees and to suppress their immune systems (Desneux et al., 2007). In addition, the sub-lethal effects of acaricides used in the hive to control varroa mites are of particular concern (Johnson et al., 2010). Acaricide levels can build up in the wax comb of colonies (Mullin et al., 2010), and low level exposure

* Corresponding author.
E-mail address: dennis.vanengelsdorp@gmail.com (D. vanEngelsdorp).
[1] Current address: Department of Biology, Kutztown University, Kutztown, PA 19530, USA.

0022-1910/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jinsphys.2011.12.011

to these products can impair a colony's ability to rear queens (Collins et al., 2004), reduce sperm viability in drones (Burley et al., 2008), and impact the development and immune response of worker bees reared in contaminated comb (Desneux et al., 2007). That being said, pesticide exposure cannot, in and of itself, explain all losses. For example, vanEngelsdorp and collaborators (2010b) found that colonies with CCD symptoms had lower levels of pesticides, specifically coumaphos, than control (without CCD) colonies.

Acaricides, which are used in honey bee colonies for the control of parasitic mites, can be divided into three categories: synthetic organics, natural products and organic acid pesticides. Synthetic organic acaricides include: (a) the pyrethroid tau-fluvalinate that kills mites by blocking the voltage-gated sodium and calcium channels (Davies et al., 2007). This product has been shown to impair queen and drone development (Rinderer et al., 1999; Haarmann et al., 2002). (b) Coumaphos, an organophosphate which inactivates acetylcholinesterase, thereby interfering with nerve signaling and function. Sub-lethal levels of coumaphos have also been shown to have a negative effect on queen development and the viability of sperm of exposed drones (Haarmann et al., 2002; Collins et al., 2004; Pettis et al., 2004; Burley et al., 2008). (c) Amitraz, a formamidine pesticide which is an octopaminergic agonist, which can impact learning and cognition in honey bees. Finally, (d) Fenpyroximate, a pyrazole acaricide that presumably kills mites through inhibition of electron transport in the mitochondria, thus interfering with energy metabolism.

Natural pesticides include thymol and menthol, both monoterpenoids which are constituents of plant-derived essential oils. Despite being naturally derived, these compounds may have negative effects on bees as both were found to be among the most toxic of all terpenoids tested on bees (Ellis and Baxendale, 1997), as well as having negative effects on a colony's ability to express hygienic behavior (Marchetti et al., 1984; Floris et al., 2004).

Organic acids include formic acid, which kills varroa mites by inhibiting electron transport in the mitochondria binding of cytochrome c oxidase (Keyhani and Keyhani, 1980). Formic acid can reduce worker longevity (Underwood and Currie, 2003) and can negatively affect brood survival (Fries, 1991).

While the sub-lethal effects of in-hive acaricides on honey bees have been demonstrated, little is known about the underlying molecular mechanisms which may cause these effects. Experiments designed to try to isolate the relevant variables contributing to colony mortality are a constant challenge, because honey bees are continually exposed to many different pathogens and environmental conditions, hampering conclusive results. To address these obstacles, a special Hawaiian (USA) population of bees that has not yet faced varroa mites or mite-associated pathogens was studied. A quantitative RT-PCR technique, covering diverse metabolic pathways, was used to determine the effects of five different in-hive acaricides on honey bees without the interference of varroa mites.

2. Materials and methods

2.1. Colony manipulations

A total of 36 colonies in one apiary in the Kona area on the Island of Hawaii, Hawaii were identified. No varroa mites had been detected in this apiary prior to this inspection. On Oct 26, 2009 selected colonies were evaluated for strength and found to be of approximately equal size based on the number of frames of brood and adult bees. A stickyboard (IPM Varroa boards, Great Lakes IPM Inc., Vestaburg, MI) was then placed in each colony and left in place for three days, confirming the apparent mite free status of these colonies. Colonies were then randomly assigned to 1 of 6 treatment groups, with each treatment group containing 6 colonies. On October 29, a sample of ½ cup of bees (~320 bees) was collected from a brood frame from each colony and placed in 70% ethanol for later nosema and varroa mite load quantification. Additionally, a 50 ml vial of adult worker bees (~100 bees) from the same brood frame was collected and placed immediately on dry ice until these bees could be stored at −80 °C until they could be processed for viral load and gene expression. Groups of colonies were then assigned a treatment and the appropriate treatment was then applied according to the manufacturer's specifications; Group 1: 2 Apistan strips (fluvalinate), Group 2: 2 CheckMite strips (coumaphos), Group 3: ApiGuard (thymol), Group 4: Mite Away (formic acid), Group5: ApiVar (amitraz), Group 6: Control, no treatment. A sticky board was placed on the floor board of each colony during the first 5 days after treatment application to ensure the varroa mite free status of the colonies. On November 12, the group receiving ApiGuard received the second dose of treatment as specified by manufacturer's directions. On December 7, 30 days after the initial treatment, samples of bees on dry ice were once again collected from the brood nest as previously described.

2.2. RNA isolation, cDNA synthesis and PCR parameters

For each colony and each collection date, a total of 30 frozen bees were homogenized together in a plastic bag with 15 ml of sterile nuclease free water after their heads were removed to avoid Polymerase Chain Reaction (PCR) Inhibitors (Boncristiani et al., 2011). From each sample, immediately after homogenization, three samples of 300 μl each were submitted to total RNA extraction, using Trizol (Invitrogen, USA) following the manufacturer's protocol. The resultant RNA pellets were resuspended in diethyl pyrocarbonate-treated water in the presence of RNase inhibitor (Invitrogen) and treated with DNase I (Invitrogen) in order to remove any contaminating DNA. The total RNA recovered from each sample was analyzed for RNA Integrity (200 ng RNA/sample) using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). All samples were classified as excellent quality, using the integrity number (RIN) that classifies RNA integrity based on a scale of 1 (completely degraded) to 10 (fully intact) based on entire electrophoretic trace of the RNA. First-strand cDNA was then synthesized by incubating 5 μg total RNA per sample in a 96-well plate with 3.9 μL of a master mix containing 50 U Superscript II (Invitrogen, Carlsbad, CA) 2 nmol DNTP mix, 2 nmol poly(dT)18, and 0.1 nmol poly(dT)(12-18) at 42 °C for 50 min followed by 15 min at 70 °C as described by (Evans et al., 2006). Primer pairs were designed to amplify 120–300 bp sections of 50 honey bee genes comprising pathogens, immune and detoxification genes (Table 1). These targets were primarily from the "BeePath" qPCR gene set (Evans, 2006), augmented by eight potential stress-response genes suggested by M. Berenbaum and G. Robinson, (*pers. comm*., Univ. Illinois). Reactions to amplify the cDNA products were conducted in 96-well plates using a Bio-Rad Icycler (Bio-Rad Corp., Hercules, CA). Fifty nano grams cDNA from each of the tested samples was used as a template for qPCR reactions using SYBR green technology (Invitrogen) following the manufacturer's protocols. The reactions were conducted under a fixed thermal protocol consisting of 5 min at 95 °C, followed by 40 cycles of a four-step protocol that involves 94 °C for 20 s, 60 °C for 30 s, 72 °C for 1 min, and 78 °C for 20 s. Fluorescence measurements were taken repeatedly during the 78 °C step. This procedure was followed by a melt-curve dissociation analysis to confirm product quality (Evans et al., 2006).

2.3. Normalization of the real-time data and statistical analysis

The amplification results were expressed as the threshold cycle number, representing the number of cycles needed to generate a

Please cite this article in press as: Boncristiani, H., et al. Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. Journal of Insect Physiology (2012), doi:10.1016/j.jinsphys.2011.12.011

Table 1
Primers used in this study.l.

Locus	Category	Gene description	F. Primer	R. Primer	Gene ID
Catalase	Detoxification	Catalase	GTCTTGGCCCAAACAATCTG	CATTCTCTAGGCCCACCAAA	AF436842.1
GSTS3	Detoxification	Glutathione S-transferase S3	TGCATATGCTGGCATTGATT	TCCTCGCCAAGTATCTTGCT	GB19254
CYP4G11	Detoxification	cytochrome P450 4G11	CAAAATGGTGTTCTCCTTACCG	ATGGCAACCCATCACTGC	GB11973
AM2446	Detoxification	Protein tyrosine phosphatase 99A ortholog	CGCGCGAGTAAGAGAAAGAG	TCGAACAAGGGAAACGAAAC	GB16234
CYP306A1	Detoxification	Cytochrome P450 306A1	CGTCGATGGGAAGGATAAAA	TCGGTGAAATATCCCGATTC	GB12311
PKA-R1	Detoxification	cAMP-dependent protein kinase type I regulatory subunit	GAAGCAATTATTCGGCAAGG	TCACCGAAACTTCCACCTTC	GB13272
CEst04	Detoxification	Esterase FE4-like (predicted)	TTTTGGGCCACGTTTACTTC	CAAATCGGTGGGTGTCTTCT	GB13591
CYP6AS14	Detoxification	Cytochrome P450 6AS14	TGAAACTCATGACCGAGACG	AAAATTTGGGCCGCTAATAAA	GB19113
PKA-C1	Detoxification	cAMP-dependent protein kinase 1	TCCATTTTTGGTCTCCTTGC	GTAAAAGCGCGAATGTGGTT	GB17175
AM12900Bredo	Detoxification	28S ribosomal RNA	TTAAGCAACCAACGCCTTTC	GGATCATGAAGCCACGAGAT	544668
AmNOS	Detoxification	Nitric oxide synthase (NOS)	TCCACTCGCAGGTACTTTCC	TCTGGAGGATCACCATTTCC	UGID:2337021
AMActin	House keeping	Actin related protein 1	TTGTATGCCAACACTGTCCTTT	TGGCGCGATGATCTTAATTT	NP 001172074.1
RPS5	House keeping	Ribosomal protein S5a	AATTATTTGGTC GCTGGAATTG	TAACGTCCAGCAGAATGTGGTA	GB11132
VGMC	Development	Vitellogenin (Vg)	AGTTCCGACCGACGACGA	TTCCCTCCCACGGAGTCC	UGID:1213462
AMHex10869	Development	Hexamerin 70b	AACTCGCTCAACTTTCCACAA	GGCTCACATAACTAACCTCACC	Gene ID: 406117
cactus	Immune	IkB transcription factor	CCTGGACTGTCTGGATGGTT	TGGCAAACCCTTTCTCAATC	GB19910
tab	Immune	Tab Tak1-binding protein	GCTATCATGCAGCTGTTCCA	ACACTGGGTCAGCCAATTTC	GB18650
PGRPSC4300	Immune	Peptidoglycan recognition protein S1	GAGGCTGGTACGACATTGGT	TTATAACCAGGTGCGTGTGC	GB15371
abaecin	Immune	Abaecin	CAGCATTCGCATACGTACCA	GACCAGGAAACGTTGGAAAC	GB18323
PGRPLC710	Immune	Peptidoglycan recognition protein LC	TCCGTCAGCCGTAGTTTTTC	CGTTTGTGCAAATCGAACAT	GB17188
PPOact	Immune	Serine protease 8	GTTTGGTCGACGGAAGAAAA	CCGTCGACTCGAAATCGTAT	GB18767
hymenopt	Immune	Hymenoptaecin	CTCTTCTGTGCCGTTGCATA	GCGTCTCCTGTCATTCCATT	GB17538
relish	Immune	NF-kappaB transcription factor relish	GCAGTGTTGAAGGAGCTGAA	CCAATTCTGAAAAGCGTCCA	Gene ID:552247
defensin1	Immune	Defensin 1	TGCGCTGCTAACTGTCTCAG	AATGGCACTTAACCGAAACG	GB19392
AmEater	Immune	NimC1, Eater-like	CATTTGCCAACCTGTTTGT	ATCCATTGGTGCAATTTGG	GB14645
domeless	Immune	Hopscotch JAK-STAT signaling pathway	TTGTGCTCCTGAAAATGCTG	AACCTCCAAATCGCTCTGTG	Gene ID:726002
defensin2	Immune	Defensin 2 (Def2)	GCAACTACCGCCTTTACGTC	GGGTAACGTGCGACGTTTTA	GB10036
PGRPSCnew	Immune	Peptidoglycan recognition protein S2	CACAAAATCCTCCGCCATT	ATGTCACCCCAACCCTTCTC	UGID:1217378
ApidNT	Immune	Apidaecin type 22	TTTTGCCTTAGCAATTCTTGTTG	GTAGGTCGAGTAGGCGGATCT	Gene ID:58585226
Bgluc19452	Immune	similar to GCN5 general control of ami no-acid synthesis 5-like 2	GGACAACCACCTTTTGAACG	AGGAGCTTCCTCTGCACTGA	Gene ID: 552646
basket	Immune	JNK MAP kinase	AGGAGAACGTGGACATTTGG	AATCCGATGGAAACAGAACG	GB16401
Dscam3–7	Immune	Down syndrome cell adhesion molecule	TTCAGTTCACAGCCGAGATG	ATCAGTGTCCCGCTAACCTG	GB15141
dorsal-1	Immune	Dorsal; NFkB transcription factor ortholoque	AAATGGTTCGCTCGTAGCAC	TCCATGATATGAGTGATGGAAA	GB19537
Nosapis	Pathogen	Nosema apis 16S ribosomal RNA gene	CAATATTTTATTGTTCTGCGAGG	TATATTTATTGTATTGCGCGTGCT	FJ789798.1
M. Pluton	Pathogen	M.pluton gene for 16S ribosomal RNA (European foulbrood disease)	ACGCCTTAGAGATAAGGTTTC	GCTTAGCCTCGCGGTCTTGCGTC	X75752
BQCV	Pathogen	Black queen cell virus	TTTAGAGCGAATTCGGAAACA	GGCGTACCGATAAAGATGGA	HQ655494.1
ABPV	Pathogen	Acute bee paralysis virus isolate GF-f1ab	ACCGACAAAGGGTATGATGC	CTTGAGTTTGCGGTGTTCCT	HM228893.1
bact774_1391	Pathogen	Generic primer for bacteria levels	CCATTTGCTTCAGGGAAGAG	CAAGCCAGCGTATGCTGTAA	
DWV	Pathogen	Deformed wing virus isolate	GAGATTGAAGCGCATGAACA	TGAATTCAGTGTCGCCCATA	AY292384.1
PlS18	Pathogen	Paenibacillus larvae ribosomal protein S18 gene	TTCACGGCTAACAAAATTAAACA	TTCGCAGAAGTTCCGGTTAC	DQ811781.1
F17 R111	Pathogen	Israel acute paralysis virus of bees	CGAACTTGGTGACTTGAAGG	GCATCAGTCGTCTTCCAGGT	EF219380.1
IAPVF1aR1	Pathogen	Israel acute paralysis virus of bees	GCGGAGAATATAAGGCTCAG	CTTGCAAGATAAGAAAGGGGG	EF219380.1
KBV	Pathogen	Kashmir bee virus	TGAACGTCGACCTATTGAAAAA	TCGATTTTCCATCAAATGAGC	AY275710.1
Acawood	Pathogen	Acarapis externus isolate B4E5 cytochrome oxidase subunit I	TCAATTTCAGCCTTTTATTCAAGA	AAAACATAATGAAAATGAGCTACAA	HQ243442.1
FungFF1R1	Pathogen	Generic primer for fungus levels	GTTAAAAAGCTCGTAGTTG	CTCTCAATCTGTCAATCCTTATT	
A. apis	Pathogen	Ascosphaera apis 28S large subunit ribosomal RNA gene	TCTGGCGGCCGGTTAAAGGCTTC	GTTTCAAGACGGGCCACAAAC	AY004344
CBPV	Pathogen	Chronic bee paralysis virus RNA-dependent RNA polymerase (RdRp) gene	CAAAATCAACGAGCCAATCA	AGTGTGAGGATCACCGGAAC	AY004344.1
SBV	Pathogen	Sacbrood virus	GGGTCGAGTGGTACTGGAAA	ACACAACACTCGTGGGTGAC	AF092924.1
qNC40sRP	Pathogen	Nosema ceranae	AGAAACTACAACAGCATCACTGGGA	AGTGAATATTCCAATTCCCAACGACTT	XM 002996328.1

fluorescent signal greater than a pre-defined threshold. In order to accurately measure the level of expression, two reference genes were used for normalization (RPS5 and β-actin). At first, a screening of the 50 different gene candidates was carried out. The cDNA samples of each treatment were combined and tested as described above. Genes whose average expression (C_T) was higher or lower than the average plus two standard deviations of the controls were selected for individual testing. For display purposes, transcript

Please cite this article in press as: Boncristiani, H., et al. Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. Journal of Insect Physiology (2012), doi:10.1016/j.jinsphys.2011.12.011

abundance values ($C_T$controls–$C_T$target) for each gene were median-normalized across each panel of genes and presented as relative red/blue-scale values using JMP 9 software (SAS Institute Inc., 2009). To evaluate the variation in gene transcript levels between different treatments a Before-After Control-Impact (BACI) design was used as a test statistic (Smith, 2002). BACI provides a way of comparing data before treatment with data obtained after treatment, as a repeated measures analysis of variance ANOVA (Bachanová et al., 2002) using colonies as replicates and the covariance structure that best suits the data (PROC MIXED, SAS Institute Inc., 2009). Each variable is measured at the start of the experiment to show existing conditions before treatment and then after a treatment. The analysis then looks at whether the change in variable measured was different between treatment groups using contrasts. p values <0.05 were considered significant after Bonferroni correction.

3. Results

3.1. Varroa mites

No mites were found in any colonies during the initial screening. One mite was found in one colony during the treatment period, indicating that mite presence remained extremely low (similar screens from areas where mites are established would find tens of mites in each colony; Boecking and Genersch, 2008).

3.2. Acaricide effects on detoxification gene expression pathways

A strong response at detoxification pathways was observed. Three acaricides were responsible for this response, Apiguard (thymol), Checkmite (coumaphos) and Mite Away (formic acid). Of all the detoxification genes screened, four were shown to have been affected, representing two immense families of important metabolic enzymes: Cytochrome p450 and protein kinases (Fig. 1). Cytochrome p450 superfamily "CYP", a large and diverse group of enzymes responsible essentially for oxidation catalysis of organic substances, had one down-regulated (cyp306a1 variant) and one up-regulated (cyp6a514 variant). For the protein kinases superfamily, up-regulation was observed on two cAMP-dependent protein kinase genes (Fig. 1; Table 2). None of the remaining detoxification genes showed a statistically significant change over the 30-day treatment period, although some did trend toward a significant change in expression ($p < 0.1$) (Table 2). For example, bees from colonies treated with Checkmite (coumaphos) showed the 28S ribosomal gene down-regulated with a p value of 0.06, suggesting that the total RNA transcription machinery could have been affected by coumaphos exposure.

Apiguard (thymol) treatment showed the strongest effect on the detoxification gene expression. Expression of four tested genes changed over the course of this experiment. Expression of two of these genes, cyp6A514 and PKAR1 was not affected by any other acaricide treatment other than the thymol treatment. Coumaphos treatment changed the expression of two genes, cyp306a1 and PKAc1, while formic acid treatment affected the expression of the PKAc1 gene only (Fig. 1). Both Apistan (tau-fluvalinate) and ApiVar (amitraz) exposure did not lead to statistically significant changes in the measured detoxification genes.

3.3. Acaricide effects on immune system gene expression

Changes in gene expression involved in immune responses were also monitored. A total of 18 immune-genes covering immune-related pathways were studied (Table 1). From the first screening, eight genes presented substantial alteration and were selected for further analysis. In the end, three genes presented significant down-regulation (Fig. 2; Table 2): (1) *Down syndrome cell adhesion molecule* (Dscam), a gene of general importance to cellular immunity and also critical for neuronal differentiation, (2) *vitellogenin*, which encodes a pleiotropic protein that affects many physiological processes including immunosenescence (Amdam et al., 2005), and (3) *basket*, an orthologue of the JUN NH_2-terminal kinase (JNK) signaling component that can activate melanization and antimicrobial and apoptotic defense mechanisms (Evans et al., 2006). The Dscam primers annealed to invariable exons 3 and 7, thus spanning the hypervariable exons 4 and 6 and potentially amplifying multiple isoforms of this gene.

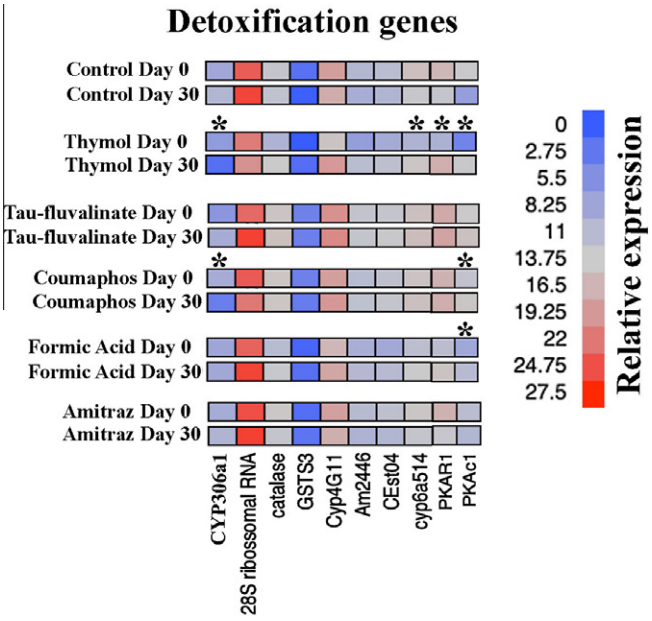

Figure 1. Heat map of detoxification gene levels in honey bee colonies treated with different acaricides from a population that had not yet received any exposure to *Varroa destructor*. cDNA levels of detoxification related genes from several pathways were quantified by Real Time PCR. Each dot represents the average of six colonies per group (30 bees/colony). Thymol, coumaphos and Formic acid treated colonies presented gene expression alterations. A Before-After Control-Impact (BACI) design was used to determine the effect of each treatment.*p values <0.05 were considered significant after Bonferroni correction.

Table 2
Honey bee gene expression altered by acaricides.

Gene	Function	Regulation (up/down)	p value
Apiguard (thymol)			
CYP306	Detoxification	Down	0.0035
CYP6a514	Detoxification	Up	0.02
pkar	Detoxification	Up	0.0165
pkac	Detoxification	Up	<0.0005
VGMC	Development	Down	<0.0015
DSC37	Immunogene	Down	<0.0005
BASK	Immunogene	Down	0.023
Apistan (tau-fluvalinate)			
Gene expression not significantly different			
Checkmite (coumaphos)			
CYP306	Detoxification	Down	0.001
pkac	Detoxification	Up	0.0445
VGMC	Development	Down	0.001
DSC37	Immunogene	Down	<0.0005
BASK	Immunogene	Down	0.002
Mite Away (formic acid)			
pkac	Detoxification	Up	0.0265
Amitraz			
Gene expression not significantly different			

Please cite this article in press as: Boncristiani, H., et al. Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. Journal of Insect Physiology (2012), doi:10.1016/j.jinsphys.2011.12.011

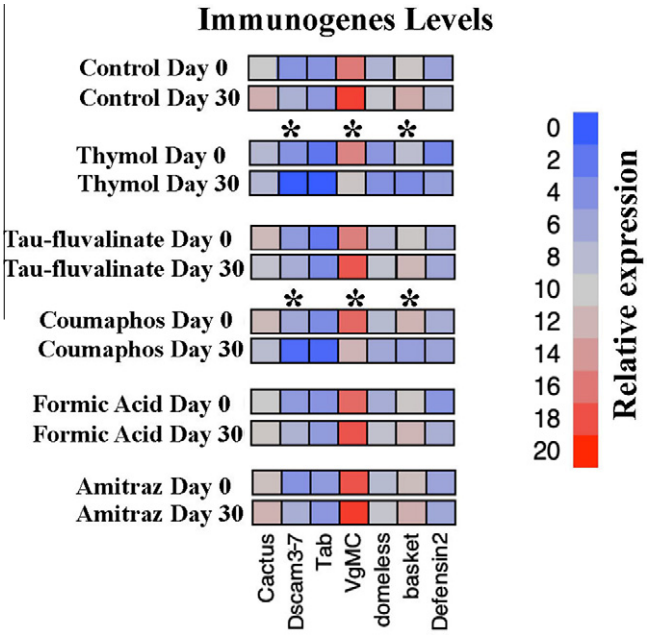

Figure 2. Heat map of selected Immuno-gene levels from honey bee colonies treated with different acaricides, from a population that had not yet been exposed to *Varroa destructor*. cDNA levels of immuno-related genes from many immuno-pathways were quantified by Real Time PCR. Each dot represents the average of six colonies per group (30 bees/colony). Apiguard and CheckMite presented some down-regulated immuno-pathways. A Before-After Control-Impact (BACI) design was used to determine the effect of each treatment.*p values <0.05 were considered significant after Bonferroni correction.

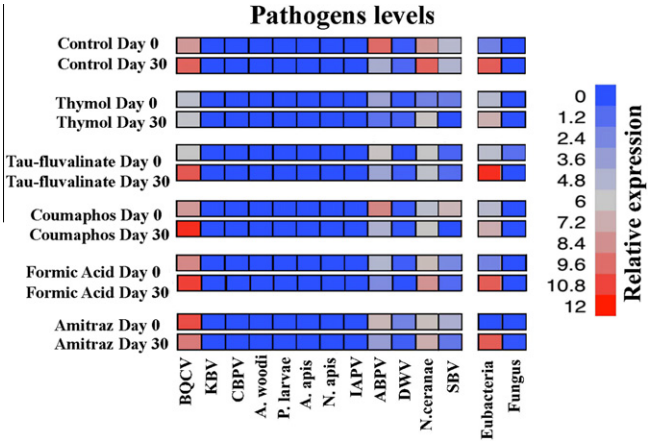

Figure 3. Heat map of pathogen levels in honey bee colonies treated with different acaricides, from a population that had not yet been exposed to *Varroa destructor*. cDNA levels of many honey bee pathogens were quantified by Real Time PCR (Black Queen Cell Virus (BQCV), Kashmir Bee Virus (KBV), Chronic Bee Paralysis Virus (CBPV), tracheal mite (*A. woodi*), American foulbrood (*P. larvae*), chalkbrood (*A. apis*), *Nosema apis*, Israeli Acute Paralysis Virus (IAPV), Acute Bee Paralysis Virus (ABPV), Deformed Wing Virus (DWV), SacBrood Virus (SBV), the microsporidian *Nosema ceranae* and using "generic" primers for estimation of the whole amount of fungus and bacteria). Each dot represents the average across six colonies per group (30 bees/colony). Low levels of pathogens were detected, confirming previous reports. KBV, CBPV, *A. woodi*, *P. larvae*, *A. apis*, *N. apis* and IAPV were not detected in any colony analyzed. Viruses generally associated with mite infestation, including DWV, IAPV, ABPV and SBV were practically absent, in sharp contrast with viruses not related with mite infestations, such as BQCV, reaffirming the importance of varroa mites in the biological cycle of those viruses. BACI analysis showed no statistical differences.

3.4. Acaricide effects on pathogen levels

A total of 12 different pathogens plus two "generic" pairs of primers used to estimate the total level of eubacteria and fungi were used to evaluate the pathogen and microorganism levels of varroa mite-free bees under the influence of in-hive acaricide treatments. Broadly, low levels of pathogens were found in all colonies investigated, consistent with the fact that they had not been exposed to mite vectors at any point, and confirming the characteristic of the region (Rose et al., 2010).

As compared to the change in pathogen load in control colonies, none of the acaricide treatments significantly changed pathogen loads in treated colonies (Fig. 3). Exposure to the acaricides thymol and coumaphos induced changes in honey bee detoxification and immune response transcripts and led to reduced levels of some pathogens. This tendency became visually evident on Fig. 3 where total eubacteria transcript levels increased over time only in controls and in bees treated with acaricides that do not initiate a detoxification and immune response (tau-fluvalinate, formic acid and amitraz). This difference was almost statistically significant on those groups (thymol $p = 0.093$ and coumaphos $p = 0.096$). A similar pattern could be observed on BQCV in the thymol group.

Honey bee pathogens such as IAPV, KBV, CBPV, and parasites, such as, *Acarapis woodi*, *Paenibacillus larvae*, *Ascosphaera apis*, and *Nosema apis* were not detected in any colonies studied. BQCV was the most prevalent pathogen, having been detected in 98.5% of all colonies tested. ABPV was also found in most colonies tested (91.4%), followed by *Nosema ceranae* (81.4%), eubacteria (75.7%), SBV (42.8%), DWV (35.7%) and fungi (0.04%). Quantitative RT-PCR analysis showed that BQCV was not only the most prevalent, but also the most abundant pathogen transcript found in all hives tested with a mean of 8.7 ± 5.1 (normalized scale), followed by *Nosema ceranae* 6.4 ± 4.7, eubacteria 6.3 ± 5.3, ABPV 5.0 ± 3.4, SBV 2.5 ± 4.2, DWV 0.5 ± 1.4 and whole fungi 0.1 ± 0.7.

4. Discussion

The varroa mite, *V. destructor*, is arguably the most serious pest of honey bees, *A. mellifera* L. Finding acaricides with minimal harmful effects to honey bees is a challenge. Honey bees are unusually susceptible to various insecticides (Atkins, 1975), as indicated by the marked reduction of genes encoding xenobiotic detoxifying enzymes found in the honey bee genome sequence (Honeybee Genome Sequencing Consortium, 2006). Insecticide toxicity is generally measured using acute contact toxicity values (LD_{50}) and toxicity studies at gene expression levels are few and restricted to biological assays for specific components (Claudianos et al., 2006). A gene expression profile analysis of honey bees historically free from *V. destructor* in the field facing in-hive acaricide treatments provides an opportunity for insight into metabolic changes caused directly by those acaricides in lieu of confounding mite impacts. This study is the first to achieve this.

4.1. Detoxification

Since honey bees are constantly exposed to different compounds in nature, the activation of detoxification pathways probably do not necessarily represent negative effect to the colony. However, overloading these detoxifying cascades by exposing bees to large quantities of pesticides, such as miticide application to colonies, potentially harms colonies by diminishing their ability to detoxify other natural or synthetic compounds.

P450 family monooxygenase genes are associated with environmental responses, including resistance to pesticides (Claudianos et al., 2006). In addition, there are some recent radiations in P450s in honey bees that are seemingly associated with the evolution of the hormonal and chemosensory processes underpinning their highly organized eusociality (Claudianos et al., 2006). Our results show alterations on gene expression levels for two p450

subfamily genes: cyp6A by thymol treatment and cyp306A by thymol and Coumaphos treatments.

CYP6s together with CYP4 group members are most commonly involved in insecticide metabolism and resistance (Claudianos et al., 2006) whereas CYP306A is highly likely to be involved in ecdysteroid biosynthesis (Niwa et al., 2004; Claudianos et al., 2006; Yamazaki et al., 2011).

Activation of P450 enzymes in honey bees challenged by acaricide exposure has been demonstrated before (Johnson et al., 2006, 2009). This activation is at least in part responsible for the rapid detoxification process shown by honey bees. In studies conducted under laboratory conditions, a synergistic effect of Coumaphos and Tau-fluvalinate on P450s activity on the detoxification process was found. Since P450 activity was detected biochemically in this study, our results for those acaricides indicate that other P450 enzymes could be involved in P450 detoxification activity rather than the CYP genes tested here; CYP4 clade (cyp4G11) and CYP6 clade (cyp6aS14). More testing will be necessary to find the main CYP gene responsible for coumaphos detoxification activity.

In this study, cyp6s14 up regulation was triggered by thymol treatment. This provides new evidence for a role of P450 activity on detoxification. It is possible that thymol is a direct or an indirect substrate for this enzyme.

The down regulation observed on cyp306A1 gene expression in bees under the influence of thymol and coumaphos is a very interesting result. Thymol, a plant-derived monoterpene phenol, and coumaphos, an organophosphate pesticide, were able to down regulate a gene associated with synthesis of one of the most important insect hormones, 20-hydroxyecdysone (20E). The down regulation of cyp306 could be involved with the protein kinase pathway alterations observed in our data (Fig 1). Protein kinase pathways are responsible for ecdysteroid synthesis control from dietary cholesterol or phytosterols via a series of hydroxylation steps (Thummel and Chory, 2002; Gilbert et al., 2011). 20E and juvenile hormone are key regulators of insect development, including the differentiation of the alternative caste phenotypes of social insects (Yamazaki et al., 2011). 20E triggers the key regulatory cascades controlling the synchronized changes in developmental pathways during molting and metamorphosis (White et al., 1997; Thummel, 2001). Uncontrolled alteration of ecdysone production could be very influential to hive sustainability, culminating in unpredictable consequences. The down regulation of cyp306A observed here could be a feedback response from high levels of 20E triggered by those chemicals, explaining the down regulation of Vitellogenin gene expression. Vitellogenin is implicated in early forager behavior and consequently shorter longevity, and also in the down regulation of some immune system pathways observed (Fig. 2; Amdam et al., 2005).

4.2. Immune system and pathogens levels

Our survey of 18 immunity-related genes revealed that acaricides may be impacting cellular immunity and immune signaling cascades. We found that the application of thymol and coumaphos to honey bee colonies resulted in the down regulation of Dscam levels after 30 days (Fig. 2; Table 2). Dscam is a multidomain extracellular recognition receptor in insects with the potential to generate thousands of isoforms via alternative splicing (Gravely et al., 2004). Distinct isoforms are expressed by neurons and hemocytes where they mediate neural wiring (Schmucker et al., 2000) and pathogen recognition (Watson et al., 2005). In honey bees, Dscam has been shown to play a role in social immunity against the Varroa mite (Navajas et al., 2008; Le Conte et al., 2011). Varroa mite-resistant bees were found to have lower expression levels of several Dscam isoforms (Le Conte et al., 2011). Thus, Dscam isoform regulation conceivably affects hygienic behavior in bees via neurons of olfaction or behavioral phenotype (Le Conte et al., 2011). In this context, the inhibition of Dscam by thymol and coumaphos treatments observed in our study may be important for compound impacts on bee behavior. Because the heads of bees were removed prior to RNA isolation, the altered Dscam expression we detected is likely to be restricted of olfaction. Alternatively, given the role of antigen recognition demonstrated in other insects, the observed Dscam down regulation may have consequences on parasite detection and removal. Further study on the roles of Dscam isoforms in honey bees is warranted before extrapolation of gene expression data to specific biological effects can be clearly made.

Basket transcription was also significantly reduced at 30 days post thymol and coumaphos application (Table 2), indicative of a potential long-term effect from the use of these acaricides on honey bee intracellular JNK signaling. The JNK pathway is triggered by extracellular stimuli like growth factors, hormones, stress, and environmental factors, with a variety of downstream effects including apoptosis, gene expression, mitosis and cell differentiation (Lodish et al., 2008). It is hard to predict consequences of alterations in such a complex signal transduction pathway, but the fact that antimicrobials were not affected (abaecin, apidaecin, defensin 1 and 2, hymenoptaecin) suggests that *basket* down-regulation by thymol and coumaphos may have consequences on functions not assessed here. Alternatively, the antimicrobial effector response may have been sustained or compensated for by other signaling pathways. In fact, we detected no acaricide impacts on Toll, JAK/STAT, or Imd immune signaling via *cactus*, *domeless*, *dorsal-1*, *relish* or *tab* expression (Table 1), supporting the unaltered immune effector response we observed.

While we detected the down regulation of genes whose products are involved in cellular (Dscam) and humoral (Basket) immunity, overall, the immune system seems unaffected by the tested acaricides. While we did not test the ability of these bees to respond to varroa mites or other disease challenges, they were supporting both viral and microspodial infections, and as such our measured changes in immune transcripts have bearing on actual abilities of individual bees to survive biological threats. However, levels of pathogens not vectored by varroa mites were not significantly affected by chemical treatments, indicating that the immune response toward these agents was not targeted by chemical treatments (Fig. 3).

Vitellogenin, a pleiotropic gene that affects many physiological processes, was also down-regulated. Its role on cellular immunity as pathogen-recognition receptors could be linked to Dscam down regulation observed in this study. It is not clear how much influence Vitellogenin has on immunity in honey bees. However, reduced Vitellogenin levels may cause immune senescence in foragers by an intricate orchestration on cellular immunity of workers triggered by behavioral stimulus and/or absence of nutrients (Amdam et al., 2005). Immune senescence is characterized by a significant reduction of hemocyte cells in foragers, therefore the low levels of Dscam could be attributed to low levels of hemocytes caused by the reduced levels of Vitellogenin found.

Vitellogenin may modulate longevity as well. *Vitellogenin* knockdown reduces the life span of honey bees in part by inducing an early shift from nursing to the short-lived forager stage (Nelson et al., 2007), suppressing juvenile hormone (Flatt et al., 2005; Guidugli et al., 2005). Acaricide-induced lifespan reduction may be very harmful, potentially compromising the sustainability of the whole colony.

This study does not necessarily show that thymol and coumaphos are harmless to honey bees. The decision to use those acaricides depends on varroa mite levels and the resulting risk from the many pathogens related with this ectoparasite, especially viruses (Chen et al., 2004a; Shen et al., 2005; Di Prisco et al., 2011). In our case, the honey bee viruses IAPV, KBV, CBPV, ABPV,

Please cite this article in press as: Boncristiani, H., et al. Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. Journal of Insect Physiology (2012), doi:10.1016/j.jinsphys.2011.12.011

DWV and SBV, which are transmitted and/or activated by varroa mite parasitism (Bowen-Walker et al., 1999; Chen et al., 2004b; Shen et al., 2005; deMiranda et al., 2010; Di Prisco et al., 2011), when detected, presented low replication levels compared to a non varroa-associated virus BQCV. The introduction of a chemical that could potentially diminish the lifespan, compromise the immune system and affect metabolic growth of honey bees, in a system already affected by pathogens would certainly impact bee health.

Acknowledgements

The authors would like to thank the entire Beltsville Bee Research Laboratory staff for their valuable support. In particular, Ms. Dawn L. Lopez provided excellent technical support and Dr. Miguel Corona gave valuable comments on the preparation of this manuscript. Additionally, we thank Gus Rouse and co-workers of Kona Queen Co and the Darcy Oishi and colleagues at the Hawaiian Department of Agriculture for great support in the field. We especially thank May Berenbaum and Gene Robinson for suggesting candidate detoxification genes important for bee health.

References

Amdam, G.V., Aase, A.L.T.O., Seehuus, S., Fondrk, M.K., Norberg, K., Hartfelder, K., 2005. Social reversal of immunosenescence in honey bee workers. Experimental Gerontology 40 (12), 939–947. doi:10.1016/j.exger.2005.08.004.

Atkins, E.L., 1975. Injury to honey bees by poisoning. In: Graham, J.M. (Ed.), The Hive and the Honey Bee. Dadant & Sons, Hamilton IL.

Bachanová, K., Klaudiny, J., Kopernický, J., Shacekimúth, J., 2002. Identification of honeybee peptide active against *Paenibacillus larvae larvae* through bacterial growth-inhibition assay on polyacrylamide gel. Apidologie 33, 259–269.

Biesmeijer, J.C., Roberts, S.P.M., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., Schaffers, A.P., Potts, S.G., Kleukers, R., Thomas, C.D., Settele, J., Kunin, W.E., 2006. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science 313 (5785), 351–354. doi:10.1126/science.1127863.

Boecking, O., Genersch, E., 2008. Varroosis – the ongoing crisis in bee keeping. Journal für Verbraucherschutz und Lebensmittelsicherheit 3 (2), 221–228. doi:10.1007/s00003-008-0331-y.

Boncristiani, H., Li, J., Evans, J., Chen, Y.P., 2011. Scientific note on PCR inhibitors in the compound eyes of honey bees, *Apis mellifera*. Apidologie 42 (4), 457–460. doi:10.1007/s13592-011-0009-9.

Bowen-Walker, P., Martin, S., Gunn, A., 1999. The transmission of deformed wing virus between honeybees (*Apis mellifera* L.) by the ectoparasitic mite *Varroa jacobsoni* Oud. Journal of Invertebrate Pathology 73 (1), 101–106.

Burley, L.M., Fell, R.D., Saacke, R.G., 2008. Survival of honey bee (Hymenoptera: Apidae) spermatozoa incubated at room temperature from drones exposed to miticides. Journal of Economic Entomology 101 (4), 1081–1087.

Chen, Y.P., Siede, R., 2007. Honey bee viruses. Advances in virus research 70, 33–80. doi:10.1016/S0065-3527(07)70002-7.

Chen, Y.P., Zhao, Y., Hammond, J., Hsu, H., Evans, J., Feldlaufer, M., 2004a. Multiple virus infections in the honey bee and genome divergence of honey bee viruses. Journal of Invertebrate Pathology 87 (2–3), 84–93.

Chen, Y.P., Pettis, J.S., Evans, J., Kramer, M., Feldlaufer, M., 2004b. Transmission of Kashmir bee virus by the ectoparasitic mite *Varroa destructor*. Apidologie 35 (4), 441–448. doi:10.1051/apido:2004031.

Claudianos, C., Ranson, H., Johnson, R.M., Biswas, S., Schuler, M.A., Berenbaum, M.R., Feyereisen, R., Oakeshott, J.G., 2006. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect Molecular Biology 15 (5), 615–636. doi:10.1111/j.1365-2583.2006.00672.x.

Collins, A.M., Pettis, J.S., Wilbanks, R., Feldlaufer, M.F., 2004. Performance of honey bee (*Apis mellifera*) queens reared in beeswax cells impregnated with coumaphos. Journal of Apicultural Research 43 (3), 128–134.

Cornman, R.S., Chen, Y.P., Schatz, M.C., Street, C., Zhao, Y., Desany, B., Egholm, M., Hutchison, S., Pettis, J.S., Lipkin, W.I., Evans, J.D., 2009. Genomic analyses of the microsporidian *Nosema ceranae*, an emergent pathogen of honey bees. PLoS Pathogens, 5(6), e1000466. doi:10.1371/journal.ppat.1000466.

Davies, T.G.E., Field, L.M., Usherwood, P.N.R., Williamson, M.S., 2007. DDT, pyrethrins, pyrethroids and insect sodium channels. IUBMB life 59 (3), 151–162. doi:10.1080/15216540701352042.

deMiranda, J.R., Cordoni, G., Budge, G., 2010. The Acute bee paralysis virus-Kashmir bee virus-Israeli acute paralysis virus complex. Journal of Invertebrate Pathology, 103 Suppl 1, S30–47. doi:10.1016/j.jip.2009.06.014.

Desneux, N., Decourtye, A., Delpuech, J., 2007. The sublethal effects of pesticides on beneficial arthropods. Annual Review of Entomology 52 (1), 81–106. doi:10.1146/annurev.ento.52.110405.091440.

Di Prisco, G., Pennacchio, F., Caprio, E., Boncristiani, H.F., Evans, J.D., Chen, Y.P., 2011. *Varroa destructor* is an effective vector of Israeli acute paralysis virus in the honeybee, *Apis mellifera*. Journal of General Virology 92, 151–155. doi:10.1099/vir.0.023853-0.

Ellis, M.D., Baxendale, F.P., 1997. Toxicity of seven monoterpenoids to tracheal mites (Acari: Tarsonemidae) and their honey bee (Hymenoptera: Apidae) hosts when applied as fumigants. Journal of Economic Entomology 90 (5), 1087–1091.

Evans, J.D., Aronstein, K.A., Chen, Y., Hetru, C., Imler, J.-L., Jiang, H., Kanost, M., Thompson, G., Zou, Z., Hultmark, D., 2006. Immune pathways and defence mechanisms in honey bees *Apis mellifera*. Insect Molecular Biology 15, 645–656. doi:10.1111/j.1365-2583.2006.00682.x.

Evans, J.D., 2006. Beepath: an ordered quantitative-PCR array for exploring honey bee immunity and disease. Journal of Invertebrate Pathology 93 (2), 135–139. doi:10.1016/j.jip. 2006.04.004.

Faucon, J.P., Mathieu, L., Ribière, M., Martel, A.-C., Drajnudel, P., Zeggane, S., Aurieres, C., Aubert, M.F.A., 2002. Honey bee winter mortality in France in 1999 and 2000. Bee World 83 (1), 14–23.

Flatt, T., Tu, M., Tatar, M., 2005. Hormonal pleiotropy and the juvenile hormone regulation of Drosophila development and life history. Bioessays 27 (10), 999–1010. doi:10.1002/bies.20290.

Floris, I., Satta, A., Cabras, P., Garau, V.L., Angioni, A., 2004. Comparison between two thymol formulations in the control of *Varroa destructor*: effectiveness, persistence, and residues. Journal of Economic Entomology 97 (2), 187–191.

Fries, I., 1991. Treatment of sealed honey bee brood with formic acid for control of *Varroa jacobsoni*. American Bee Journal 131 (5), 313–314.

Fries, I., 2010. *Nosema ceranae* in European honey bees (*Apis mellifera*). Journal of Invertebrate Pathology 103, S73–S79. doi:10.1016/j.jip. 2009.06.017.

Gilbert, L.I., Rybczynski, R., Warren, J.T., 2011. Control and biochemical nature of the ecdysteroidogenic pathway. Annual Review of Entomology 47 (1), 883–916. doi:10.1146/annurev.ento.47.091201.145302.

Gravely, B.R., Kaur, A., Gunning, D., Zipursky, S.L., Rowen, L., Clemens, J.C., 2004. The organization and evolution of the Dipteran and Hymenopteran Down syndrome cell adhesion molecule (Dscam) genes. RNA (New York, NY), 10(10), 1499–1506.

Guidugli, K., Nascimento, A., Amdam, G., Barchuk, A., Omholt, S., Simoes, Z., Hartfelder, K., 2005. Vitellogenin regulates hormonal dynamics in the worker caste of a eusocial insect. FEBS Letters 579 (22), 4961–4965. doi:10.1016/j.febslet.2005.07.085.

Haarmann, T., Spivak, M., Weaver, D., Weaver, B., Glenn, T., 2002. Effects of fluvalinate and coumaphos on queen honey bees (Hymenoptera: Apidae) in two commercial queen rearing operations. Journal of Economic Entomology 95 (1), 28–35.

Haynes, K.F., 1988. Sublethal effects of neurotoxic insecticides on insect behavior. Annual Review of Entomology 33, 149–168. doi:10.1146/annurev.en.33.010188.001053.

Honeybee Genome Sequencing Consortium. (2006). Insights into social insects from the genome of the honeybee Apis mellifera Nature, 443 (7114), 931–949.

Johnson, R.M., Wen, Z., Schuler, M.A., Berenbaum, M.R., 2006. Mediation of pyrethroid insecticide toxicity to honey bees (Hymenoptera: Apidae) by cytochrome P450 monooxygenases. Journal of Economic Entomology 99 (4), 1045–1050.

Johnson, R.M., Pollock, H.S., Berenbaum, M.R., 2009. Synergistic interactions between in-hive miticides in *Apis mellifera*. Journal of Economic Entomology 102 (2), 474–479.

Johnson, R., Ellis, M., Mullin, C., 2010. Pesticides and honey bee toxicity – USA. Apidologie 41 (3), 312–331.

Keyhani, J., Keyhani, E., 1980. EPR study of the effect of formate on cytochrome C oxidase. Biochemical and Biophysical Research Communications 92 (1), 327–333.

Le Conte, Y., Alaux, C., Martin, J.-F., Harbo, J.R., Harris, J.W., Dantec, C., Séverac, D., Cros-Arteil, S., Navajas, M., 2011. Social immunity in honeybees (*Apis mellifera*): transcriptome analysis of varroa-hygienic behaviour. Insect Molecular Biology 20 (3), 399–408. doi:10.1111/j.1365-2583.2011.01074.x.

Lodish, H., Berk, A., Zipursky, S., 2008. Molecular Cell Biology (6th ed.). New York, NY.

Marchetti, S., Barbattini, R., D'Agaru, M., 1984. Comparative effectiveness of treatments used to control *Varroa jacobsoni* Oud. Apidologie 15 (4), 363–378. doi:10.1051/apido:19840401.

Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., vanEngelsdorp, D., Pettis, J.S., 2010. High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PLoS ONE, 5(3), e9754. doi:10.1371/journal.pone.0009754.

Navajas, M., Migeon, A., Alaux, C., Martin-Magniette, M.L., Robinson, G.E., Evans, J.D., Cros-Arteil, S., Crauser, D., LeConte, Y., 2008. Differential gene expression of the honey bee *Apis mellifera* associated with *Varroa destructor* infection. BMC Genomics 9, 301. doi:10.1186/1471-2164-9-301.

Nelson, C.M., Ihle, K.E., Fondrk, M.K., Page, R.E., Amdam, G.V., 2007. The gene vitellogenin has multiple coordinating effects on social organization. PLoS Biology, 5(3), e62. doi:10.1371/journal.pbio.0050062.

Neumann, P., Carreck, N.L., 2010. Honey bee colony losses. Journal of Apicultural Research 49 (1), 1–6.

Niwa, R., Matsuda, T., Yoshiyama, T., Namiki, T., Mita, K., Fujimoto, Y., Kataoka, H., 2004. CYP306A1, a cytochrome P450 enzyme, is essential for ecdysteroid biosynthesis in the prothoracic glands of *Bombyx* and *Drosophila*. Journal of Biological Chemistry 279 (34), 35942–35949. doi:10.1074/jbc.M404514200.

Pettis, J.S., Collins, A., Wilbanks, R., 2004. Effects of coumaphos on queen rearing in the honey bee, *Apis mellifera*. Apidologie 35, 605–610.

Please cite this article in press as: Boncristiani, H., et al. Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. Journal of Insect Physiology (2012), doi:10.1016/j.jinsphys.2011.12.011

Rinderer, T.E., DeGuzman, L.I., Lancaster, V.A., Delatte, G.T., Stelzer, J.A., 1999. Varroa in the mating yard:: I. The effect sof Varroa jacobsoni and Apistan on drone honey bees. American Bee Journal 139 (2), 134–139.

Rose, R., Hayes, J., Pettis, J., vanEngelsdorp, D., Rennich, K., Snyder, R., Andee, M., Patel, N., Roccasecca, K., Rice, N., Lopez, D., Evans, J., Levi, V., 2010. The national honey bee disease and pest survey: 2009-2010 pilot study. American Bee Journal 150 (11), 1033–1035.

SAS Institute Inc., 2009. Using JMP 9. SAS Institute Inc., Cary, NC.

Schmucker, D., Clemens, J.C., Shu, H., Worby, C.A., Xiao, J., Muda, M., Dixon, J.E., Zipursky, S.L., 2000. *Drosophila* Dscam is an axon guidance receptor exhibiting extraordinary molecular diversity. Cell 101 (6), 671–684. doi:10.1016/S0092-8674(00)80878-8.

Shen, M., Yang, X., Cox-Foster, D., Cui, L., 2005. The role of varroa mites in infections of Kashmir bee virus (KBV) and deformed wing virus (DWV) in honey bees. Virology 342 (1), 141–149. doi:10.1016/j.virol.2005.07.012.

Smith, E., 2002. BACI design. In: El-Shaarawi, A.H., Piegorsch, W.W. (Eds.), Encyclopedia of Environmetrics, vol. 1. John Wiley & Sons Ltd, Chichester, pp. 141–148.

Thompson, H.M., 2003. Behavioural effects of pesticides in bees–their potential for use in risk assessment. Ecotoxicology 12 (1–4), 317–330.

Thummel, C.S., 2001. Molecular mechanisms of developmental timing in *C. elegans* and *Drosophila*. Developmental cell 1, 453–465.

Thummel, C.S., Chory, J., 2002. Steroid signaling in plants and insects—common themes, different pathways. Genes & Development 16 (24), 3113–3129.

Underwood, R.M., Currie, R.W., 2003. The effects of temperature and dose of formic acid on treatment efficacy against *Varroa destructor* (Acari: Varroidae), a parasite of *Apis mellifera* (Hymenoptera: Apidae) Experimental and Applied Acarology, 29(3-4), 303–313.

vanEngelsdorp, D., Hayes, J. Jr., Underwood, R. M., Pettis, J., 2008. A Survey of honey bee colony losses in the U.S., fall 2007 to spring 2008. PLoS ONE, 3(12), e4071.

vanEngelsdorp, D., Evans, J.D., Saegerman, C., Mullin, C., Haubruge, E., Nguyen, B.K., Frazier, M., Frazier, J., Cox-Foster, D., Chen, Y.P., Underwood, R., Tarpy, D.R., Pettis, J.S., 2009. Colony collapse disorder: a descriptive study. PLoS ONE, 4(8), e6481. doi:10.1371/journal.pone.0006481.

vanEngelsdorp, D., Meixner, M.D., 2010. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. Journal of Invertebrate Pathology, 103 Suppl 1, S80–95. doi:10.1016/j.jip.2009.06.011.

vanEngelsdorp, D., Hayes Jr., J., Underwood, R.M., Pettis, J.S., 2010a. A survey of honey bee colony losses in the United States, fall 2008 to spring 2009. Journal of Apicultural Research 49 (1), 7–14. doi:10.3896/IBRA.1.49.1.03.

vanEngelsdorp, D., Speybroeck, N., Evans, J.D., Nguyen, B.K., Mullin, C., Frazier, M., Frazier, J., Cox-Foster, D., Chen, Y.P., Tarpy, D.R., Haubruge, E., Pettis, J.S., Saegerman, C., 2010b. Weighing risk factors associated with bee colony collapse disorder by classification and regression tree analysis. Journal of Economic Entomology 103 (5), 1517–1523.

Watson, F.L., Püttmann-Holgado, R., Thomas, F., Lamar, D.L., Hughes, M., Kondo, M., Rebel, V.I., Schmucker, D.L., 2005. Extensive diversity of Ig-superfamily proteins in the immune system of insects. Science 309 (5742), 1874–1878.

White, K.P., Hurban, P., Watanabe, T., Hogness, D.S., 1997. Coordination of Drosophila metamorphosis by two ecdysone-induced nuclear receptors. Science 276 (5309), 114–117.

Yamazaki, Y., Kiuchi, M., Takeuchi, H., Kubo, T., 2011. Ecdysteroid biosynthesis in workers of the European honeybee Apis mellifera L Insect Biochemistry and Molecular Biology, 41(5), 283–293. doi:10.1016/j.ibmb.2011.01.005.

Please cite this article in press as: Boncristiani, H., et al. Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. Journal of Insect Physiology (2012), doi:10.1016/j.jinsphys.2011.12.011